British Journal of Pharmacology (2000) 129, 533–540
www.nature.com/bjp

The effects of heparin and related molecules upon the adhesion of human polymorphonuclear leucocytes to vascular endothelium *in vitro*

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1 The effects of an unfractionated heparin preparation (Multiparin), a low molecular weight heparin preparation (Fragmin) and a selectively O-desulphated derivative of heparin lacking anticoagulant activity, have been investigated for their effects on the adhesion of human polymorphonuclear leucocytes (PMNs) to cultured human umbilical vein endothelial cells (HUVECs) *in vitro*. The effect of poly-L-glutamic acid, a large, polyanionic molecule was also studied.

2 Unfractionated heparin (50–1000 U ml^{-1}), the O-desulphated derivative (0.3–6 mg ml^{-1}) and the low molecular weight heparin (50 U–1000 U ml^{-1}) all inhibited significantly the adhesion of ^{51}Cr labelled PMNs to HUVECs stimulated with interleukin-1β (IL-1β; 10 U ml^{-1}), bacterial lipopolysaccharide (LPS; 2.5 μg ml^{-1}) or tumour necrosis factor-α (TNF-α; 125 U ml^{-1}) for 6 h, whereas poly-L-glutamic acid had no effect. In addition, the three heparin preparations in the same concentration range inhibited significantly the adhesion of f-met-leu-phe-stimulated PMNs to resting HUVECs.

3 The effects of unfractionated heparin upon the expression of adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and E-selection were also investigated, as were the effects of unfractionated heparin upon adhesion of human PMNs to previously stimulated HUVECs. Heparin had little effect upon levels of expression of these adhesion molecules on stimulated HUVECs. However, a profound effect upon PMN adhesion to previously stimulated HUVECs was demonstrated using the same preparation, suggesting that inhibition of adhesion molecule expression is not a major component of the described inhibitory effects of heparin.

4 Pre-incubation of PMNs with heparin followed by washing inhibited their adhesion to HUVECs, under different conditions of cellular activation, implying that heparin can bind to these cells and exert its anti-adhesive effects even when not directly present in the system.

5 These observations would suggest that both heparin and a low molecular weight heparin are capable of inhibiting adhesion of human PMNs to endothelial cells, an effect not dependent solely upon the polyanionic nature of these molecules, nor dependent upon their ability to act as anticoagulants.

British Journal of Pharmacology (2000) **129,** 533–540

Keywords: Heparin; low molecular weight heparin; O-desulphated heparin; adhesion; polymorphonuclear leucocytes; endothelium; polyanionic charge; anticoagulant

Abbreviations: ELISA, enzyme linked immunosorbant assay; f-met-leu-phe, formyl methionine leucyl phenylalanine; GAG, glycosaminoglycan; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IgG, immunoglobulin G; IL-1β, interleukin-1β; LPS, lipopolysaccharide; N-CAM, neural cell adhesion molecule; PAF, platelet activating factor; PBS, phosphate buffered saline; PGA, poly-L-glutamic acid; PMN, polymorphonuclear leucocyte; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF-α, tumour necrosis factor-α

Introduction

There is considerable interest in finding mechanisms to prevent the excessive trafficking of inflammatory cells from the circulation into tissues during inflammatory responses (e.g. Wardlaw *et al*., 1996). As an early event preceding transendothelial migration, leucocyte-endothelial cell adhesion is now recognized as an important event, and the mechanisms initiating this process are increasingly well understood (e.g. Brown & Lindberg, 1996). However, little is known about the mechanisms which physiologically limit this process, although it is well recognized that the endothelial cells lining the blood vessels are coated with the glycosaminoglycan (GAG) heparan sulphate (Tyrrell *et al*., 1995) and that disruption of this layer modulates the adhesion of leucocytes (Giuffrè *et al*., 1997; Sivaram *et al*., 1995).

GAGs, particularly heparin, are better known for their anticoagulant actions although there is now considerable evidence showing that these molecules possess a wide array of other activities (Tyrrell *et al*., 1995). For example, previous studies have shown that GAGs can be anti-inflammatory (e.g. Ley *et al*., 1991; Matzner *et al*., 1984; Sasaki *et al*., 1993) and we have described the ability of a range of GAGs to inhibit the trafficking of inflammatory cells into the airway *in vivo* induced by both platelet activating factor (PAF) and antigen/antibody interactions in suitably sensitized animals (Seeds *et al*., 1993; 1995). However, the mechanisms underlying these anti-inflammatory effects are as yet unknown, but evidence continues to accumulate which demonstrates that heparin can

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modulate the adhesion of leucocytes to vascular endothelium (e.g. Bazzoni *et al*., 1992; Diamond *et al*., 1995, Silvestro *et al*., 1994; Xie *et al*., 1997).

Heparin has been shown to inhibit f-met-leu-phe-activated neutrophil adherence to resting endothelial cells (Bazzoni *et al*., 1992), and another study demonstrated that heparin and certain partially desulphated derivatives, were able to inhibit non-activated neutrophil adherence to PAF stimulated endothelial cells (Silvestro *et al*., 1994). However, this latter effect was observed only when the glycosaminoglycans (GAGs) were present in the neutrophil suspension, not when added to the endothelial cells. The results of these studies imply that heparin may have a direct effect upon leucocytes, as opposed to endothelial cells.

The aim of the present study was to investigate the effects of an unfractionated heparin preparation, a low molecular weight heparin and a selectively O-desulphated derivative of heparin, devoid of anticoagulant activity, upon the *in vitro* adhesion of human PMNs to cultured human endothelial cells, when added to the endothelial cells rather than to the PMN suspension. The leucocyte does not play an active role in this early stage, as demonstrated by Patel and colleagues (1991), who have shown that formaldehyde-fixed neutrophils can adhere to stimulated endothelial cells. The effect of poly-L-glutamic acid (PGA) was also studied, in order to ascertain whether any observed actions of heparin and related molecules might be attributable simply to their being polyanionic molecules.

In addition, the effects of unfractionated heparin upon the expression of the adhesion molecules ICAM-1 and E-selectin were studied, along with the effect of heparin upon PMN adhesion to previously activated endothelial cells, in order to determine whether heparin inhibits adhesion by affecting the upregulation of endothelial adhesion molecules.

Our study was also extended to investigate the effects of heparin upon neutrophil dependent adhesion, i.e. the adhesion observed when stimulated neutrophils come into contact with resting endothelial cell monolayers. Additionally, the direct actions of heparin upon neutrophil adhesive functions were studied by pre-incubating these cells with heparin then washing the cells before examining their adhesion to HUVEC monolayers under different conditions of cellular activation.

Methods

Preparation of PMNs

Peripheral venous blood was collected from healthy volunteers into tubes containing anticoagulant (1 volume in 10, acid citrate dextrose) and erythrocytes were removed by mixing the citrated blood with an equal volume of a 6% dextran solution (Hespan) and sedimenting at room temperature for 30–40 min. PMNs were then isolated from the white cell rich plasma by density-dependent centrifugation on Percoll gradients (55% on 70% on 81%), the PMNs being collected from the 70%:81% interface. Contaminating erythrocytes were removed by hypotonic lysis (0.83% ammonium chloride) and cell pellets were washed three times with modified Hank's balanced salts solution (Ca^{2+} and Mg^{2+} free). Cells were then radiolabelled with ^{51}Cr by incubating cell pellets for 1 h at 37°C in 150 μL aqueous sodium chromate (37 MBq ml^{-1}), then washing three times with modified Hank's balanced salts solution. Cells were resuspended at a concentration of 1×10^6 cells ml^{-1} in Hank's balanced salts solution (unmodified), ready for use in the assay. PMN populations isolated in this manner were found to be more than 98% viable, as measured by trypan blue exclusion and consisted of not less than 95% neutrophils. The process of radiolabelling did not affect cell viability.

Adhesion assay-stimulated HUVECs

The adhesion assay used is based upon a method described by Kyan-Aung *et al*. (1991). In brief, cryopreserved HUVECs (TCS Ltd., U.K.) were cultured in endothelial cell basal medium (MCDB 131) supplemented with foetal bovine serum (2%), hydrocortisone (1 ng ml^{-1}), gentamicin (50 μg ml^{-1}), amphotericin-B (50 ng ml^{-1}) and human epidermal growth factor (10 ng ml^{-1}). Cells were passaged by trypsinisation (0.025% trypsin with 0.01% EDTA) and were grown to confluency in the central wells of flat-bottomed 96-well plates in a volume of 200 μl culture medium per well. For adhesion assays, cells were used at the fifth passage.

Cells were stimulated in culture medium with interleukin-1β (IL-1β; 0–100 U ml^{-1}), bacterial lipopolysaccharide (LPS; 0–100 μg ml^{-1}) or tumour necrosis factor-α (TNF-α; 0–1000 U ml^{-1}) for 6 h, by the addition of 22 μl of a solution of stimulus at ten times the final concentration required in the well. Concentrations which effected large, submaximal responses were selected for use in the inhibitor experiments.

In the inhibitor experiments, monolayers of HUVECs were stimulated for 6 h as before, with IL-1β (10 U ml^{-1}), LPS (2.5 μg ml^{-1}) or TNF-α (125 U ml^{-1}) in the absence and presence of heparin (Multiparin; 50–1000 U ml^{-1}; anticoagulant activity approximately 180 U mg^{-1}), a low molecular weight heparin (Fragmin; 50–1000 U ml^{-1}; anticoagulant activity in the range 125–180 U mg^{-1}) or a corresponding amount of O-desulphated heparin (0.3–6 mg ml^{-1}). The effects of poly-L-glutamic acid (PGA; 1 mg ml^{-1}) were investigated also.

Following stimulation, monolayers were washed with warm Hank's Balanced Salts Solution to remove stimuli and inhibitors before the addition of 200 μl radiolabelled PMN suspension to each well (2×10^5 cells per well). Plates were then incubated for 30 min at 37°C. Non-adherent cells were removed by gentle aspiration and washing with warm Hank's Balanced Salts Solution and the adherent cells in each well were lysed with 1% Nonidet-P40, placed in scintillation vials and counted on a γ-counter. PMN viability was unaffected during the course of the assay and HUVEC monolayers retained confluency as determined by microscopic examination prior to cell lysis. Results were treated as follows:

% adhesion of PMNs was calculated as:

$$\frac{(\text{counts per minute in sample from well}/\text{total counts added to well})}{} \times 100$$

% inhibition of adhesion was calculated as:

$$[1 - (\delta \text{ in presence of drug})/(\delta \text{ in absence of drug}] \times 100$$

where δ = (% adhesion in cytokine stimulated wells) – (% adhesion in control wells) {n.b. control wells were those which had not been stimulated by cytokines but to which radiolabelled PMNs were added in order to ascertain basal levels of adhesion to HUVECs}.

Data were analysed using analysis of variance followed by a modified *t*-test (Dunnett's test).

Adhesion assay-stimulated PMNs

In these experiments, 200 μl of the PMN suspension were added to wells of unstimulated HUVECs and plates were incubated for 5 min at 37°C in the absence and presence of the

heparin preparations (as before). As a PMN stimulus, 22 μl of a 10^{-5} M solution of fMLP was then added to the wells, yielding a final concentration of 10^{-6} M fMLP; a concentration which had been found previously to cause submaximal PMN activation, as measured by homotypic aggregation. Plates were incubated for a further 30 min at 37°C, and were then treated as described before.

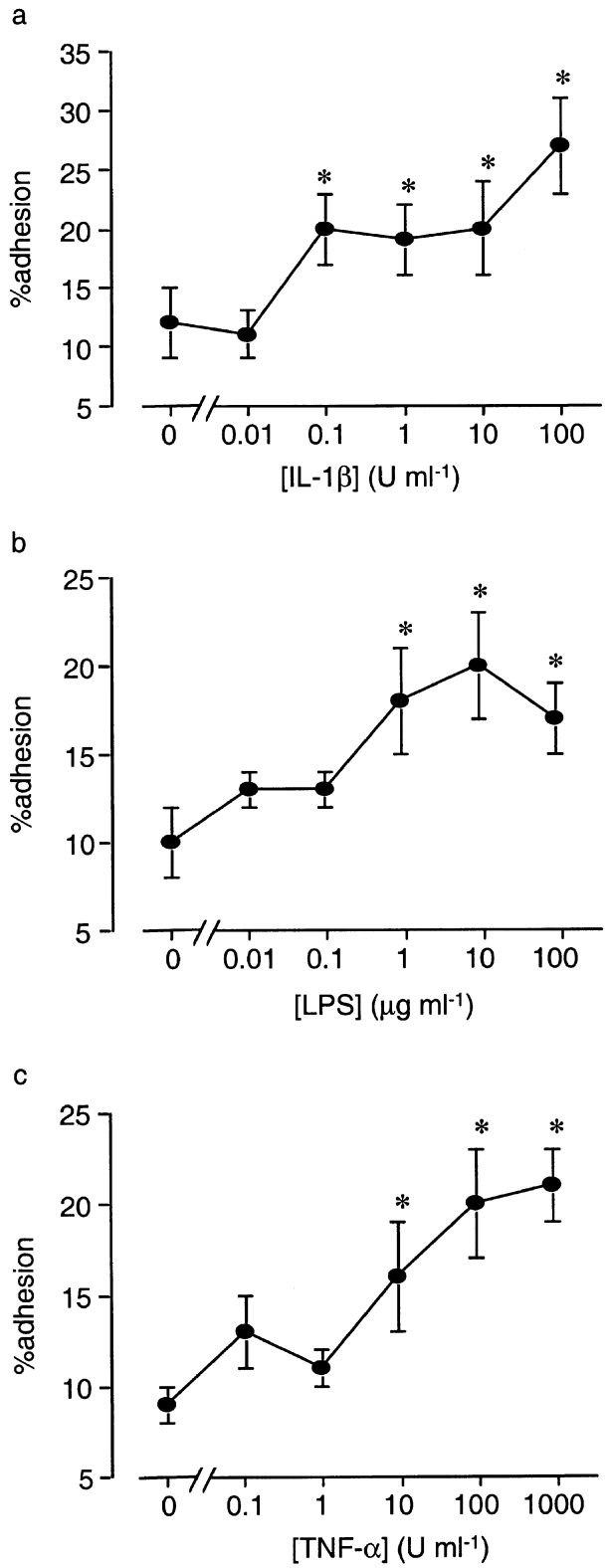

Figure 1 Adhesion of human PMNs to stimulated HUVECs. HUVECs were stimulated for 6 h with (a) IL-1β (0–100 U ml^{-1}), (b) LPS (0–100 μg ml^{-1}) or (c) TNF-α (0–1000 U ml^{-1}). ^{51}Cr-labelled PMNs ($n=6$) were added for 30 min and adhesion was quantified by γ-counting lysates of adherent cells and calculating the percentage of cells added which had adhered. Results are expressed as mean values$\pm$s.e.mean from experiments performed in triplicate. *Statistical significance ($P<0.05$) is indicated.

ELISA assays and pre-stimulated HUVEC adhesion assays

ELISA assays were based upon a protocol supplied by R&D Systems Ltd. Briefly, HUVEC monolayers were prepared and some wells were stimulated for 6 h as before (with IL-1β 10 U ml^{-1}, LPS 2.5 μg ml^{-1} or TNF-α 125 U ml^{-1}), in the absence and presence of unfractionated heparin (Multiparin, 50–1000 U ml^{-1}). Stimuli and heparin were removed by washing three times with a blocking solution, which consisted of 5% w v^{-1} low-fat powdered milk in phosphate buffered saline (PBS) in order to eliminate non-specific protein binding in the assay. Monolayers were incubated for 1 h at room temperature with primary monoclonal antibody (mouse anti-human ICAM-1 IgG (clone BBIG-I1), E-selectin IgG (clone BBIG-E4) or control mouse IgG_1), then were washed thoroughly with blocking solution before application of the secondary antibody (goat anti-mouse IgG-horseradish peroxidase conjugate). Monolayers were again incubated for 1 h at room temperature, then were washed thoroughly with PBS before addition of TMB substrate system (3,3′,5,5′-tetramethylbenzidine). After 10–15 min incubation at room temperature, the reaction was quenched by inclusion of 1 M H_2SO_4 (aq.) and plates were analysed colorimetrically at 450 nm in an Anthos HT III microplate reader. Expression of ICAM-1 or E-selectin in heparin treated wells was calculated as a percentage of that found in wells which had not been

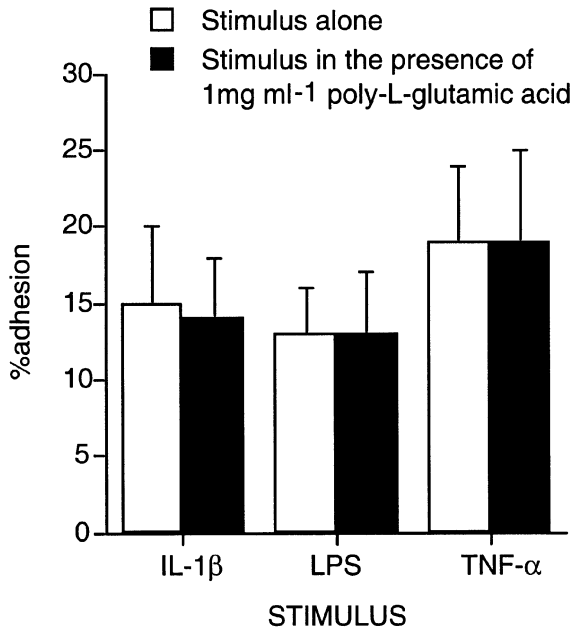

Figure 2 Effect of poly-L-glutamic acid upon human PMN adhesion to stimulated HUVECs. HUVECs were stimulated for 6 h with (a) IL-1β (10 U ml^{-1}), (b) LPS (2.5 μg ml^{-1}), or (c) TNF-α (125 U ml^{-1}), in the absence and presence of poly-L-glutamic acid 1 mg ml^{-1} ^{51}Cr-labelled PMNs ($n=6$) were added for 30 min and adhesion was quantified by γ-counting lysates of adherent cells and calculating the percentage of cells added which had adhered. Data are expressed as mean values$\pm$s.e.mean from experiments performed in triplicate. No differences were observed in the magnitude of adhesion responses elicited by the three stimuli, at the concentrations used ($P>0.05$) and poly-L-glutamic acid had no effect upon adhesion in these assays ($P>0.05$).

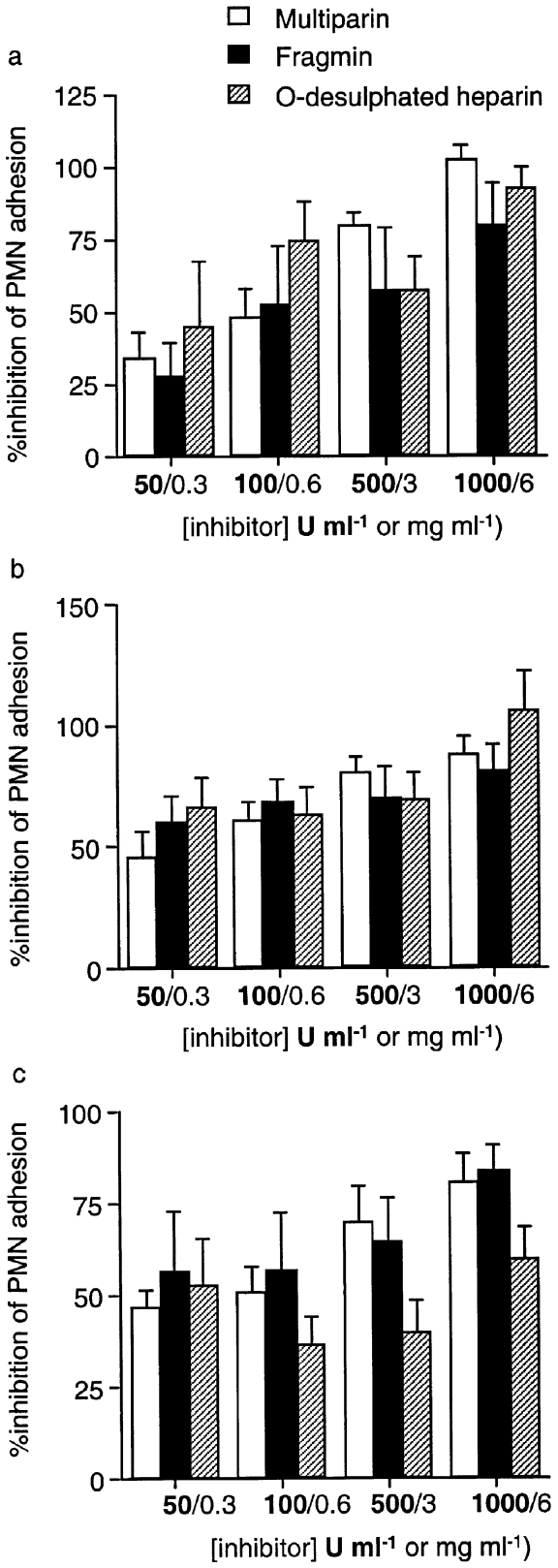

Figure 3 Inhibition of adhesion of human PMNs to stimulated HUVECs. HUVECs were stimulated for 6 h with (a) IL-1β (10 U ml^{-1}), (b) LPS (2.5 μg ml^{-1}), or (c) TNF-α (125 U ml^{-1}), in the absence and presence of the heparin preparations (0–1000 U ml^{-1} Multiparin or Fragmin, 0–6 mg ml^{-1} O-desulphated heparin). ^{51}Cr-labelled PMNs ($n=6$) were added for 30 min and adhesion was quantified by γ-counting lysates of adherent cells and comparing values with those obtained from unstimulated wells. Data are expressed as mean values $\pm$ s.e. mean from experiments performed in triplicate. All concentrations tested of the three heparin preparations inhibited adhesion when compared to control wells ($P<0.05$).

treated with heparin. Background values (obtained from wells which had been treated in the same manner but without addition of primary antibody) were subtracted from all test values. Data were analysed using analysis of variance followed by a modified *t*-test (Dunnett's test).

Additionally, some HUVEC monolayers were stimulated for 6 h without heparin. Monolayers were then washed and ^{51}Cr-labelled PMNs were added to wells in the absence and presence of heparin (Multiparin 50–1000 U ml^{-1}). Plates were treated in the same manner as described for the original adhesion assay. Adhesion was calculated as a percentage of that found in wells which had not had heparin added with the PMNs. Data were again analysed using analysis of variance followed by a modified *t*-test (Dunnett's test).

Pre-incubation experiments

In some experiments, radiolabelled PMNs were incubated for 20 min at room temperature with different concentrations of an unfractionated heparin preparation (Multiparin, 0–1000 U ml^{-1}). Pellets were then washed with Hank's balanced salts solution (modified), resuspended as before and applied to HUVEC monolayers. PMNs were either stimulated with fMLP as described above, or were not stimulated, but were added to HUVECs which had been previously incubated for 6 h with interleukin-1β (10 U ml^{-1}), bacterial lipopolysaccharide (2.5 μg ml^{-1}) or tumour necrosis factor-α (125 U ml^{-1}) and the stimuli removed by washing, as before. Plates were incubated for 30 min at 37°C, non-adherent cells removed and adherent cells quantified as before.

Reagents

Cryopreserved HUVECs and all culture media were obtained from TCS Ltd. (Milton Keynes, Bucks, U.K.); Aqueous sodium chromate (^{51}Cr) solution from Amersham Life Science, (U.K.); Percoll, Hank's balanced salts solutions, phosphate buffered saline, horseradish peroxidase linked goat anti-mouse IgG, TMB liquid substrate system, acid citrate dextrose, Nonidet-P40, poly-L-glutamic acid, cytokines (recombinant, human), fMLP and bacterial (*E. Coli*) LPS (serotype 0111:B4) from Sigma-Aldrich Company Ltd. (U.K.); Mouse anti-human ICAM-1 IgG, mouse anti-human E-selectin IgG and control mouse IgG$_1$ from R & D Systems Ltd. (U.K.); Hespan from Du Pont Pharmaceuticals Ltd. (Letchworth, Herts, U.K.); Multiparin from CP Pharmaceuticals Ltd. (Wrexham, North Wales, U.K.); Fragmin from Pharmacia Laboratories Ltd. (Milton Keynes, Bucks, U.K.); O-desulphated heparin was a gift from Dr T. Kennedy, Carolinas Medical Health Care Foundation, (Charlotte, N.C., U.S.A.).

Results

Pretreatment of confluent HUVECs for 6 h with IL-1β (0.01–100 U ml^{-1}), LPS (0.01–100 μg ml^{-1}) or TNF-α (0.1–1000 U ml^{-1}) caused concentration-dependent increases in the ability of radiolabelled PMNs to adhere to these cells (Figure 1). In a further experiment, it was found that IL-1β (10 U ml^{-1}), LPS (2.5 μg ml^{-1}) and TNF-α (125 U ml^{-1}) upregulated significantly the adhesion of radiolabelled PMNs to HUVECs to a similar extent (Figure 2). Therefore, these concentrations of stimuli were used in subsequent inhibitor experiments, mean upregulation of adhesion elicited being 9.6$\pm$1.48% (basal) to 34.45$\pm$5.24% (IL-1β, 10 U ml^{-1}),

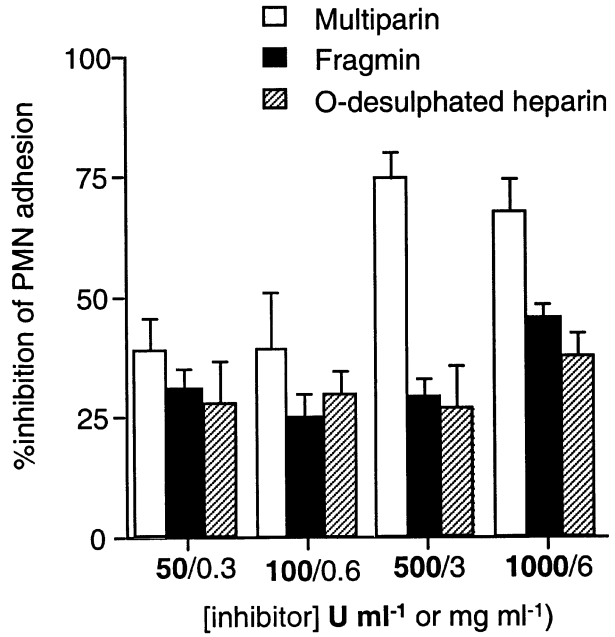

Figure 4 Inhibition of formyl-methionine-leucylphenylalanine induced PMN adhesion to HUVECs. ^{51}Cr-labelled human PMNs were added to cultured HUVECs, in the absence and presence of the substances under investigation (Multiparin or Fragmin, 0–1000 U ml^{-1}; O-desulphated heparin, 0–6 mg ml^{-1}). After 5 min, fMLP (10^{-6} M) was added to wells and plates were incubated for 30 min at 37°C. Adherent cells were lysed and γ-counted. Results from five separate experiments, each performed in triplicate, are expressed as mean inhibition of adhesion ± s.e. mean. *Statistical significance ($P<0.05$) is indicated.

29.45 ± 3.85% (LPS, 2.5 μg ml^{-1}) and 30.75 ± 3.48% (TNF-α, 125 U ml^{-1}).

Unfractionated heparin (Multiparin) and the low molecular weight heparin (Fragmin) both inhibited PMN adhesion to endothelial cells stimulated with I1-β, LPS, or TNF-α. This effect was shared by the non-anticoagulant, O-desulphated derivative of heparin (Figure 3).

However, poly-L-glutamic acid 1 mg ml^{-1} did not inhibit PMN adhesion to cytokine stimulated endothelial cells (6 ± 10%, −9 ± 18% and 4 ± 8% inhibition; when HUVECs were stimulated with IL-1β, 10 U ml^{-1}; LPS 2.5 μg ml^{-1}; TNF-α 125 U ml^{-1}; respectively; (Figure 2)).

In addition, the unfractionated heparin preparation Multiparin, the low molecular weight heparin preparation Fragmin and the non-anticoagulant, O-desulphated heparin inhibited significantly the adhesion of PMNs stimulated with 10^{-6} M fMLP to unstimulated HUVECs (Figure 4), levels of adhesion increasing from 7.40 ± 1.50% (basal) to 25.38 ± 4.44% in wells exposed to this concentration (10^{-6} M) of fMLP.

IL-1β (10 U ml^{-1}), LPS (2.5 μg ml^{-1}) and TNF-α (125 U ml^{-1}) all upregulated significantly the expression of both ICAM-1 and E-selectin over 6 h, when compared to control (unstimulated) wells. ICAM-1 expression in unstimulated wells increased from (OD450) 0.380 ± 0.039 to 0.911 ± 0.059, 0.866 ± 0.077 and 1.073 ± 0.084 in wells stimulated with IL-1β, LPS or TNF-α respectively. Similarly, E-selectin expression increased from (OD450) zero in unstimulated wells to 0.543 ± 0.043, 0.447 ± 0.043 and 0.472 ± 0.049 in wells stimulated with IL-1β, LPS or TNF-α respectively.

Unfractionated heparin had a small but significant effect upon the expression of ICAM-1 on HUVECs in response to these concentrations of IL-1β, LPS or TNF-α. No effect upon E-selectin expression on IL-1β or TNF-α stimulated

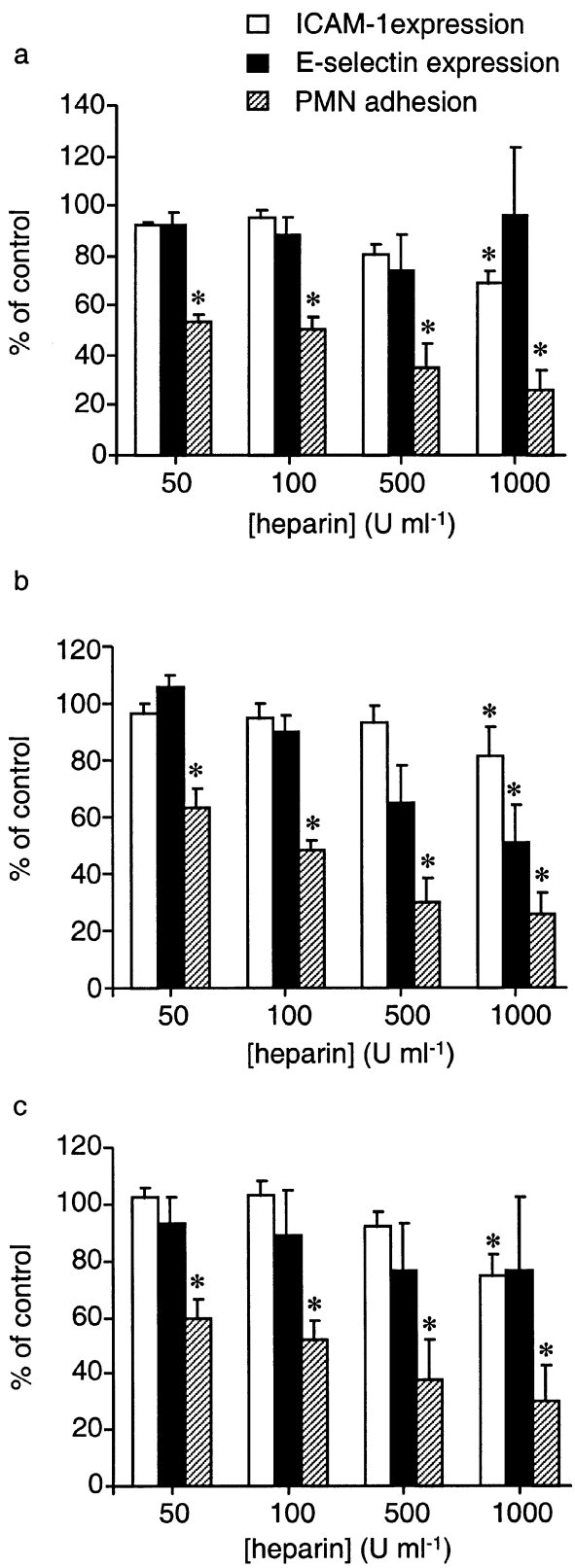

Figure 5 Effect of heparin upon adhesion molecule upregulation. HUVECs were stimulated for 6 h with (a) IL-1β (10 U ml^{-1}), (b) LPS (2.5 μg ml^{-1}), or (c) TNF-α (125 U ml^{-1}), in the absence and presence of unfractionated heparin (Multiparin). Expression of the adhesion molecules ICAM-1 (open bars) and E-selectin (closed bars) were studied using an ELISA technique. The adhesion of ^{51}Cr-labelled human PMNs in the absence and presence of unfractionated heparin (Multiparin), to HUVECs previously stimulated in the absence of heparin is also shown (hatched bars). Adhesion molecule expression and PMN adhesion were calculated as per cent of control wells which did not receive heparin. Data are expressed as mean values ± s.e. mean from six experiments performed in triplicate. *Statistical significance ($P<0.05$) is indicated.

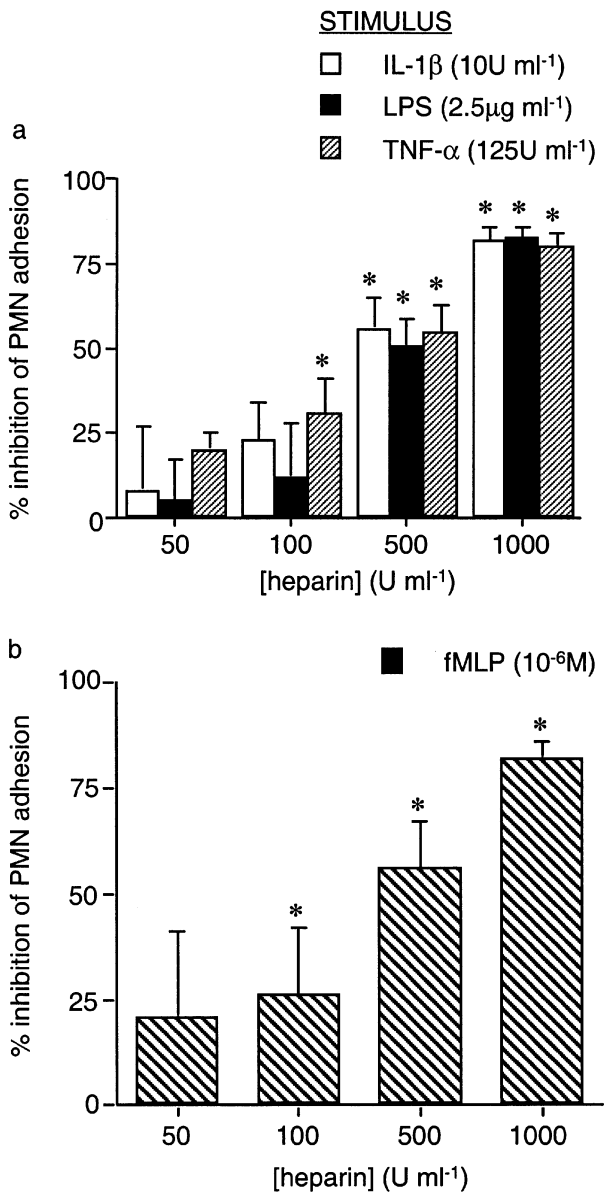

Figure 6 Inhibition of PMNs pre-incubated with heparin, to HUVECs under different conditions of cell activation. ^{51}Cr-labelled human PMNs were incubated with unfractionated heparin for 20 min before being washed. (a) PMNs were then added to HUVECs which had been previously stimulated for 6 h with IL-1β (10 U ml^{-1}), LPS (2.5 μg ml^{-1}) or TNF-α (125 U ml^{-1}). (b) PMNs were added to HUVECs and incubated for 5 min before the addition of fMLP (10^{-6} M). Plates were incubated for 30 min at 37°C. Adherent cells were lysed and γ-counted. Results from six separate experiments, each performed in triplicate, are expressed as mean inhibition of adhesion $\pm$ s.e.mean. *Statistical significance ($P<0.05$) is indicated.

cells was observed, although again, the highest concentration of heparin had a small effect upon LPS stimulated cells. (Figure 5).

However, unfractionated heparin had a potent inhibitory effect upon the adhesion of radiolabelled PMNs to HUVECs pre-stimulated with IL-1β (10 U ml^{-1}); LPS (2.5 μg ml^{-1}) or (TNF-α 125 U ml^{-1}), when added to the HUVECs immediately prior to addition of the PMN suspension. (Figure 5).

An alternative experimental protocol was adopted in order to investigate the effects of heparin on granulocyte function. Unfractionated heparin (Multiparin), when incubated with PMNs prior to their addition to HUVECs, inhibited significantly the adhesion of these cells to HUVECs which had been previously activated by stimulation with IL-1β (10 U ml^{-1}), LPS (2.5 μg ml^{-1}) or TNF-α (125 U ml^{-1}) for 6 h (Figure 6a). Similarly, the adhesion of PMNs stimulated with fMLP (10^{-6} M) to unstimulated HUVECs was inhibited by pre-incubating the leucocytes with heparin (Figure 6b).

Discussion

Our results provide evidence that an unfractionated heparin preparation can profoundly inhibit the adhesion of unstimulated PMNs to endothelial cells that have been stimulated with cytokines (IL-1β or TNF-α) or the bacterial product LPS, for 6 h. Previous studies have reported that f-met-leu-phe activated neutrophil adhesion to resting endothelial cells was inhibited by heparin (Bazzoni *et al.*, 1992), and that certain partially desulphated derivatives of heparin were also able to inhibit non-activated neutrophil adhesion to endothelial cells stimulated by PAF or thrombin (Silvestro *et al.*, 1994). However, in these latter experiments, inhibition was only observed when the GAGs were present in the neutrophil suspension and not when added to the endothelial cells. Our results contrast with those of Silvestro *et al.* (1994), as in the first part of our study, the GAGs were added to the endothelial cell preparation, but not the PMN suspension. The reason for this discrepancy is not clear, although in our studies cytokines and LPS were used as stimuli rather than PAF and thrombin and we were considering ICAM-1 and E-selectin dependent adhesion rather than a P-selectin-mediated process. However, it has been shown that thrombin can induce ICAM-1 expression on endothelial cells after as little as one minute's incubation, *via* a mechanism which is independent of *de novo* protein synthesis, as confirmed by Northern blotting (Sugama *et al.*, 1992). It is therefore possible that ICAM-1 was also present on the thrombin-stimulated endothelial cells used in the studies of Silvestro *et al.* (1994), as indeed we have found to be the case in our assays using cytokines and LPS as stimuli. In later parts of our study, we found that, in accordance with the results of Silvestro *et al.* (1994), the presence of heparin in the PMN suspension did indeed reduce the adhesion of these cells to activated HUVECs, the stimuli in this case being cytokines and LPS as endothelial stimuli, or in support of the findings of Bazzoni *et al.* (1992) fMLP as a PMN stimulus.

In our studies, we found that co-incubation of heparin with stimulated endothelial cells inhibits adhesion substantially without having a significant impact upon the expression of the adhesion molecules ICAM-1 and E-selectin on the endothelial cell surface, in that only a weak inhibitory effect upon the expression of these molecules was observed and only at the highest concentrations. Additionally, we found that heparin can inhibit PMN adhesion to HUVECs which had been pre-treated with stimuli, i.e. to cells upon which adhesion molecules were already present.

It is known that heparin binds the leucocyte adhesion molecule Mac-1 (Diamond *et al.*, 1995), an effect which is likely to be responsible for heparin's inhibitory effects upon stimulated PMN adhesion, as observed in part of our study. However, as the heparin was removed from the system prior to the addition of the PMN suspension in most of our experiments with stimulated HUVECs, the only heparin present to interact with PMN adhesion molecules was that which had bound to the endothelium. Indeed, a greater inhibitory effect was observed in the experiments where the heparin was present with the PMNs on the stimulated endothelial cells, an effect which is likely to be at least partly due to heparin interactions with the PMN surface. None-

theless, the results of our experiments suggest clearly that GAGs affect endothelial cell function as well as PMN function and it is possible that endothelial adhesion molecules are being bound by heparin, preventing adhesive interactions with their ligands on the leucocyte. It has been demonstrated that heparin is able to bind directly to neural cell adhesion molecule (N-CAM, an adhesion molecule present on neurones), *via* a heparin-binding domain located on the second immunoglobulin domain (Lindahl *et al*., 1994). It is therefore possible that a similar interaction occurs with other immunoglobulin-like adhesion molecules such as ICAM-1, which may result in a reduced adhesion in the presence of heparin. However, despite structural congruencies between the selectins, it is established that heparin is unable to bind to E-selectin (Koenig *et al*., 1998) in the manner which it binds P-selectin (Skinner *et al*., 1991), so if ICAM-1 binding does occur, one would expect only a partial inhibition of PMN adhesion to be attainable. Specific binding studies need to be carried out in order to investigate this point further.

Two studies have described an inhibition of leucocyte 'rolling' (a step essential for subsequent adhesion and extravasation) in the mesenteric vasculature of the rabbit, by intravenous administration of various GAGs including heparin. Sulphation was deemed to be essential for this effect and the higher the degree of sulphation, the greater the inhibitory effect observed (Ley *et al*., 1991; Tangelder & Arfors, 1991). Negative charge was not found to be a requisite for this inhibitory effect of GAGs and indeed, Tangelder & Arfors (1991) found that protamine sulphate, which is positively charged, was also able to inhibit leucocyte 'rolling'. Our study would support the idea that the ability of heparin to reduce PMN adhesion to vascular endothelium is not dependent solely upon its negative charge, as no inhibition was observed with poly-L-glutamic acid in our assays. The present results also support our previous *in vivo* observations that poly-L-glutamic acid does not modify PAF (Sasaki *et al*., 1991; Seeds *et al*., 1993) or allergen (Seeds *et al*., 1995) induced eosinophil infiltration into the lungs of experimental animals. Furthermore, our results with poly-L-glutamic acid would suggest that simple removal of free Ca^{2+} (upon which adhesion is dependent), or covalent binding of peptide cytokines, are not the predominant mechanisms in operation, as poly-L-glutamic acid should similarly be capable of these actions.

The ability of unfractionated heparin to inhibit PMN adhesion is shared by a low molecular weight heparin (Fragmin) and by an O-desulphated derivative of heparin. The latter observation is of particular interest as this molecule lacks anticoagulant activity (Fryer *et al*., 1997), suggesting that the inhibitory effect of GAGs on cell adhesion is not related to the well known effect of heparin of inhibition of the coagulation cascade (Jaques, 1979). We have previously reported that O-desulphated heparin is also able to inhibit allergen induced eosinophil infiltration to the lungs of guinea pigs (Seeds *et al*., 1998), suggesting that anticoagulant activity is not essential for the ability of heparin to inhibit cell trafficking *in vivo*. Our results support earlier observations that derivatives of heparin lacking anticoagulant activity inhibit other inflammatory processes such as delayed hypersensitivity reactions (Sy *et al*., 1983).

Whereas it is well established that heparin can bind to endothelium, this possibly being one mechanism by which blood vessel patency is maintained, there is less evidence suggesting functionally important consequences of the binding of heparin to the surfaces of inflammatory cells. Here, we have shown that pre-incubation of PMNs followed by extensive washing, nevertheless prevents the PMNs from adhering to vascular endothelial cells in culture, both when the endothelium is stimulated and when the PMNs are stimulated. These results suggest that a tight binding of heparin to the PMN surface also occurs which is irreversible under these experimental conditions and which causes prolonged interference with adhesive functions of these cells.

In conclusion, we have provided evidence that heparin and two related drugs can inhibit the adhesion of PMNs to endothelial cells induced by a variety of stimuli *via* a mechanism which does not involve inhibition of ICAM-1 or E-selectin expression on endothelial cells, but which probably involves the binding of cellular adhesion molecules or their counterligands. Additionally, our results suggest that this effect of heparin is independent of its polyanionic nature, unrelated to its anticoagulant actions and involves interference with the adhesive processes of both neutrophils and endothelial cells.

We wish to thank the B.B.S.R.C. for the funding of R. Lever. We also thank Dr T. Kennedy for donation of the O-desulphated heparin and the Carolinas Medical Healthcare Foundation for sponsorship of the work leading to the development of O-desulphated heparin.

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(Received July 20, 1999
Revised October 25, 1999
Accepted November 12, 1999)